THE RESPONSES OF MALE SALIENTIA TO HUMAN CHORIONIC GONADOTROPIC HORMONE

James C. Knepton, Jr.
Wesleyan College

Robbins, Parker, and Bianco (1947) have demonstrated that male *Xenopus laevis* Daudin react to the chorionic gonadotropic hormone by the emission of spermatozoa. Samson (1950) studied the variation in sensitivity of male *Rana pipiens* to chorionic gonadotrophin. Other investigators have similarly studied the reaction of various salientia. Bhaduri (1950) has an excellent summarization of work done in this field.

The present investigation was designed to study comparatively the responses of males of five native species of Florida salientia to the human chorionic gonadotropic hormone. The species selected for this experiment represent five families of Salientia. Studies were made with animals collected throughout the year (calendar year, 1950) in order to evaluate any seasonal effects. Correlative histological studies of the testes of the experimental animals were also undertaken.

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Materials and Methods

During the calendar year of 1950, male frogs were collected each month in the vicinity of Gainesville, Florida. From January to September the collections were made by the writer and from September until the end of the year five males of each species being studied were furnished by Mr. McConkey. The number of specimens of each species collected by the writer is given in Table I, the data being roughly indicative of the availability of each species.

TABLE I

Number of males of native Salientian species collected in 1950.

Species	Jan.	Feb.	Mar.	April	May	June	July	Aug.
Scaphiopus h. holbrookii	10	0	6	7	7	6	0	5
Bufo t. terrestris	1	0	14	23	23	34	4	14
Hyla c. cinerea	3	0	2	8	4	18	0	6
Rana pipiens sphenocephala	6	0	5	10	7	8	0	1
Microhyla carolinensis	0	0	0	2	1	21	8	19

Collecting was done at night for *Scaphiopus h. holbrookii* (Harlan), *Bufo t. terrestris* (Bonnaterre), *Hyla c. cinerea* (Schneider), and *Rana pipiens sphenocephala* (Cope). *Microhyla carolinensis* (Holbrook) was collected during the daytime.

Following each collection the individual frogs were placed in separate jars covered with a gauze top.

The frogs were measured for snout-vent length with a millimeter ruler, to the nearest millimeter, and were weighed with a trip balance to the nearest tenth of a gram.

The commercial chorionic gonadotropic hormone was used for this investigation in preference to a hormone-containing substance such as urine of pregnant women. The pure hormone has the very distinct advantage of eliminating a number of variables: the amount of hormone is known and the purity of the hormone is relatively constant.

Each package of human chorionic gonadotropic hormone contained a 10 cc. rubber-stoppered, multidose vial with 5,000 International Units of dehydrated hormone and an ampule filled with 10 cc. saline solution. When the dehydrated hormone was diluted, each cc. of aqueous solution contained 500 I. U. of chorionic gonadotropic hormone.

The commercial chorionic gonadotropic hormone was checked against the chorionic gonadotropic hormone international standard which is produced by the United States Pharmaceutical Reference Standards, New York City. The same results, both qualitatively and quantitatively, were obtained from frogs injected with the standard hormone and from those injected with the commercial hormone.

The injection of the frogs with the chorionic gonadotropic hormone was usually done a day or two after collection. Urine from each frog was inspected to determine whether spermatozoa were present before injection with the hormone. The urine samples were obtained by a method of "milking". "Milking" is accomplished by holding the frog in the hand with his head in the palm and the area of his cloaca held between the thumb and forefinger. Urine is then forced from the frog's bladder with the thumb and forefinger.

A 1 cc. syringe with a 27-gauge needle was used for injection purposes. While the dosages varied they were easily controlled as the original dilution resulted in 50 I. U. of chorionic gonadotropic hormone in each 0.1 cc. solution. The injections were made subcutaneously into the dorsal lymphatic sac of the salientians.

Following injection, each frog was returned to his jar. Urine samples were taken approximately one-half hour after injection and subsequently thereafter, usually at half-hour intervals when convenient, until spermatozoa were observed. With each series of injections, parallel observations were made on an uninjected control frog of the same species.

Each month the testes from an injected and control frog were removed and fixed in Bouin's fluid and later prepared for histological study. The paraffin method and an alcohol series were used in preparing the tissues. The 10-micron sections of testis were stained with iron hematoxylin and eosin and mounted with balsam. For final observations the oil immersion objective was used. The length of the spermatozoa in the testis of each species of frog was measured from the prepared slides with a Bausch and Lomb filar micrometer.

Results

Scaphiopus holbrookii holbrookii (Harlan). Family Pelobatidae. With the exception of the month of February when no specimens of this toad were found, males of this species were easily collected from January to July. Throughout July no specimens were found although an intensive search was made. In August collections were adequate for experimental purposes (see Table 1).

The adult males ranged in snout-vent length from 42 to 71 millimeters. The variations in weight were from 6 to 30 grams.

Forty-five specimens were injected with amounts of chorionic gonadotropic hormone ranging from 25 to 250 I. U. At approximately 30 minute intervals urine samples were examined for discharged spermatozoa until 36 hours following injection. The examinations were in vain. Under the conditions of this experiment spermatozoa were not discharged. An explanation of this negative result, which apparently involves the biological effect of atmospheric pressure, is being prepared for presentation elsewhere.

In injected and control Spadefoot Toads the most advanced stage of spermatogenesis found in abundance from March until May was the leptotene stage. From May until November all stages could be found in relatively the same abundance. The average length of the head of the spermatozoa of this species is 6.0 microns.

Bufo terrestris terrestris (Bonnaterre). Family Bufonidae. Male specimens of the Southern Toad were easily collected from March until September at the edges of ponds, lakes and small streams. The group used for tests varied in snout-vent length from 45 to 71 millimeters and weighed from 7 to 27 grams.

One hundred and four male specimens were injected with from 12.5 to 200 I. U. of the chorionic gonadotropic hormone. One hundred and two specimens reacted positively to the hormone by

discharging spermatozoa into the urine. Two specimens reacted negatively. Spermatozoa were not found in the urine samples from the controls. If spermatozoa were present in the urine, they always appeared in large numbers. Southern Toads injected with 50 to 200 I. U. of the chorionic gonadotropic hormone reacted by demonstrating nervousness along with the ejection of spermatozoa into the urine. Spermatozoa were first observed in urine samples as early as 25 minutes and as late as 149 minutes after injection.

Throughout the year, all stages of spermatogenesis were observed in the testes of both injected and control animals. In addition, there was an abundance of spermatozoa detached from the vacuolated Sertoli cells of the injected specimens. In the testes of the control frogs, it was observed that during August, September, and October spermatogenic figures in the pachytene and diakinesis stages are more frequent than those of the other stages. Among the controls it was also noted that spermiogenesis occurs the entire year and that spermatogonia are few in May and June.

The spermatozoa of *Bufo t. terrestris* averages 10.9 microns in length of head.

Hyla cinerea cinerea (Schneider). Family Hylidae. Male specimens of *Hyla c. cinerea* were collected during the months of January, March, June and August, 1950. The adult male specimens ranged in snout-vent length from 28 to 57 millimeters. They varied in weight from 1 to 9 grams.

The doses of chorionic gonadotropic hormone injected into male specimens of *Hyla c. cinerea* varied from 12.5 to 250 I. U. Spermatozoa were discharged by thirty of the thirty-four individuals. Spermatozoa were first observed in urine samples as early as 41 minutes and as late as 261 minutes following injection. This reaction did not appear in the controls. In addition to exhibiting nervousness, the injected frogs turned from their natural green color to a dark green and their hind legs turned black.

Prepared sections of the testes from injected and control specimens exhibited the same characteristics. The testes are yellow and average 10 to 12 millimeters in length. During each month of the experiments with this frog (January, March, June, and August) spermatogenic figures were present. Meiotic figures in the first division were abundant in August. In April and May, stages of the second meiotic division were more abundant.

Spermatozoa were always present in the lumina of the seminiferous tubules of the testes from injected specimens. The Sertoli cells when vacuolated had no spermatozoa attached to them.

The length of the head of the spermatozoon of *Hyla c. cinerea* averages 9.0 microns.

Rana pipiens sphenocephala (Cope). Family Ranidae. This species was found along the banks of small streams in the cooler periods of the year. During the warmer months, April to October, they were present at the banks of large bodies of water.

The snout-vent lengths for adult males of this species ranged from 47 to 72 millimeters. Their weights varied from 7 to 28 grams.

Thirty-two of the thirty-five specimens of the Southern Leopard Frog reacted positively to amounts of chorionic gonadotropic hormone varying from 25 to 250 I. U. In positive cases, abundant spermatozoa were discharged 27 to 167 minutes following injection with the hormone. Besides ejecting spermatozoa after injection, the medial side of the hind legs of this species turned black. Urine from the controls of this species contained no spermatozoa.

The testis of *Rana pipiens sphenocephala* is yellow and measures approximately 10 to 11 millimeters long and 3 to 4 millimeters in diameter. The individual seminiferous tubule is relatively small and filled with spermatogenic cells and spermatozoa. Various stages of spermatogenesis were present all year in both injected and control frogs.

The lumina of the seminiferous tubules in the testes of injected frogs were filled with spermatozoa detached from vacuolated Sertoli cells. The lumina of the control frogs were empty because spermatozoa were attached to the Sertoli cells. During January the walls between the seminiferous tubules of both injected and control specimens were thickened by the presence of many interstitial cells. During other months in which tests were made the number of interstitial cells was greatly reduced.

The average length of the head of a spermatozoon of this species was found to be 13.0 microns.

Microhyla carolinensis (Holbrook). Family Brevicipitidae. The first intensive collecting for this species was done in April and specimens were obtained from then until October (see Table I). The male Narrow-Mouth Toads varied in snout-vent lengths from 21 to 31 millimeters and in weight from 0.6 to 2.6 grams.

Forty-five specimens of this species were injected with amounts of chorionic gonadotropic hormone varying from 12.5 to 50 I. U. Forty-one of the Narrow-Mouth Toads reacted by discharging spermatozoa from 45 to 161 minutes after injection with the hormone. The toads reacted to the hormone in another manner besides discharging spermatozoa. After injection with any dosage of chorionic gonadotropic hormone, their bodies would move spasmodically and they apparently had no control of any muscular movement. Spermatozoa were found in the urine of injected Narrow-Mouth Toads in large quantities but they did not occur in the urine of the controls.

In relation to others studied, the testis of this species is relatively small. Sections of testis from a Narrow-Mouth Toad collected in April showed very few spermatogenic cells beyond the preleptotene and leptotene stages. From May until September the sections of testes demonstrated the abundant occurrence of all stages of spermatogenesis. A noticeable decline in the number of cells in advance meiotic stages was evidenced from the sections of testis of a toad collected in September. In all the testes removed from injected toads spermatozoa were detached from the vacuolated Sertoli cells. Sections of testes of control toads showed spermatozoa attached to non-vacuolated Sertoli cells.

The head of a sperm cell of *Microhyla carolinensis* averages 9.0 microns in length.

Discussion

The percentages of positive results obtained by injecting the various toads and frogs with chorionic gonadotropic hormone are shown in Table 2. These percentages are derived from healthy specimens that were injected with non-toxic doses of hormone. The non-conformities are discussed below.

Scaphiopus h. holbrookii does not react positively to the chorionic gonadotropic hormone under the conditions of the present experiments. The possible reasons for the negative reaction will be considered in a forthcoming report.

Two injected males of *Bufo t. terrestris* did not react, presumably due to the toxic nature of large dosages. It may naturally be that a heavy frog needs a large dose and a light frog a relatively smaller dose.

TABLE II

Percentages of native male Salientia discharging spermatozoa after being injected with human chorionic gonadotropic hormone.

Species	Total number of frogs injected.	Number of adult frogs in good health injected with less than toxic but more than minimal dosage.	Number of negatives from column 2.	Percent positive.
Scaphiopus h. holbrookii	45	45	45	0
Bufo t. terrestris	104	102	0	100
Hyla c. cinerea	34	30	0	100
Rana p. sphenocephala	35	32	0	100
Microhyla carolinensis	45	43	2	95.3

Four individuals of *Hyla c. cinerea* injected with the chorionic gonadotropic hormone did not discharge spermatozoa. It is believed that these specimens were possibly given too large a dose of hormone (2 received 200 I. U.; 2 received 100 I. U.) for the emission of spermatozoa. However a single specimen also injected in April with 100 I. U. reacted positively. But it took this specimen 261 minutes to react which was the longest reaction time observed during the entire study of this frog. The same dosage, causing both negative and positive responses, may indicate that 100 I. U. is a critical level and that lower doses should be used.

The explanation for a specimen of *Rana pipiens sphenocephala* which reacted negatively in May, may depend on the fact that its coelom was heavily parasitized by roundworms. It is thought that a specimen which reacted negatively in June did not respond because the dose injected was too small.

Four male specimens of *Microhyla carolinensis* did not react to the chorionic gonadotropic hormone. Two probably died because of the toxic effects of an overabundance of the hormone (50 I. U.) and for undeterminable reasons the other two did not react. As in the case with specimens of *Hyla c. cinerea,* where 100 I. U. is the critical maximum dosage, 50 I. U. may be the threshold value for *Microhyla carolinensis* since one specimen injected with that amount in April reacted positively. The critical maximum dose of chorionic

gonadotropic hormone is thought to be that dose which produces positive and negative reactions.

Secondary effects caused by the injection of chorionic gonadotropic hormone into specimens of Salientia used in this investigation were (1) changes in color of various regions of the body and (2) an interference with muscular coordination. As found in this study, the hind legs of *Rana pipiens sphenocephala* turned black when injected with the hormone. Color change produced in males of this species is thought by the writer to be reliable for indicating the presence of the chorionic gonadotropic hormone in their bodies. The color change exhibited by *Hyla c. cinerea* after injection with the hormone was a turning from a natural green to a very dark green. Some specimens turned black on their head, hind legs, and back as a result of hormone injection. The color change in this species is not reliable as an indicator of the hormone. There were detectable color changes on the bodies of the members of the other species under investigation that were neither as definable nor as frequent as those described for the above two. It is interesting to note that Bisnet (1935) reports apparent body color changes in fish following injection with a chorionic gonadotropic hormone-containing substance.

The other secondary reaction, the discordant movement of the body, produced by almost any dosage of hormone was observed in specimens of *Scaphiopus h. holbrookii, Hyla c. cinerea, Bufo t. terrestris,* and *Microhyla carolinensis.*

The mechanism of spermatozoa release in the testes of the salientia investigated was found to be as previously determined by former workers. The principal investigators, DeRobertis *et al* (1946) and Burgos and Mancini (1948), state that the release of spermatozoa is accomplished by the progressive vacuolization of the Sertoli cells and subsequent rupture of the apical ends of the cells.

A histological study of sections of testes of all injected specimens showed spermatozoa detached from the Sertoli cells and free in the lumina of the seminiferous tubules. In contrast, the sections of testes from controls showed almost all spermatozoa attached to the Sertoli cells. The lumina of the seminiferous tubules of the controls were virtually empty.

The reaction of injected *Scaphiopus h. holbrookii* to the hormone is peculiar. Even though the Sertoli cells rupture and release the

spermatozoa, sperm cells are not obtained in the urine samples.

The sections of testes indicate a seasonal spermatogenic cycle for *Scaphiopus h. holbrookii, Hyla c. cinerea,* and *Microhyla carolinensis. Bufo t. terrestris* and *Rana pipiens sphenocephala* had spermatogenesis occurring the entire period of study. Burgos and Mancini (1948) reported that in another species of Bufonidae, *Bufo arenarum,* spermatogenesis is likewise continuous.

Summary

1. Individuals of five species of Salientia, representing five families native to Florida, were injected with commercial human chorionic gonadotropic hormone during 1950. Continuing monthly collections made available males of the following forms: Pelobatidae, *Scaphiopus h. holbrookii;* Bufonidae, *Bufo t. terrestris;* Hylidae, *Hyla c. cinerea;* Ranidae, *Rana pipiens sphenocephala;* and Brevicipitidae, *Microhyla carolinensis* most months of the year.

2. The primary reaction to various doses of the chorionic gonadotropic hormone by the species of toads and frogs was the discharge of spermatozoa.

3. No male individuals of *Scaphiopus h. holbrookii* discharged spermatozoa following an injection of the hormone. Males of the other four species reacted positively.

4. Secondary reactions to the chorionic gonadotropic hormone were the changing of the skin color and neuro-muscular action that was demonstrated by kicking of the limbs and uncontrolled blinking of the eyes.

5. A histological study of the testes from a control and an injected frog of each species was made for each month in which animals were tested. Section of testes from control and injected *Scaphiopus h. holbrookii, Hyla c. cinerea,* and *Microhyla carolinensis* show that an annual spermatogenic cycle occurs. Continuous production of spermatozoa is characteristic of *Rana pipiens sphenocephala* and *Bufo t. terrestris.* The lumina of the seminiferous tubules of the control animals were empty, whereas those of the injected animals were filled with spermatozoa, detached from the Sertoli cells.

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